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Tissue Differences in Fragile X Mosaics: Mosaicism in Blood Cells May Differ Greatly From Skin

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The fragile X mutation is diagnosed from the structure of the FMR1 gene in blood cell DNA. An estimated 12 to 41% of affected males are mosaics who carry both a "full mutation" allele from which there is no gene expression and a "premutation" allele which has normal gene expression. We compared the DNA in blood cells and skin fibroblasts from four mosaic fragile X males to see if there was a difference in the relative amounts of premutation and full mutation alleles within the tissues of these individuals. Two of these males showed striking differences in the ratio of premutation to full mutation in different tissues while the other two showed only slight differences. These observations conform with the widely accepted hypothesis that the fragile X CGG repeat is unstable in somatic tissue during early embryogenesis. Accordingly, the mosaicism in brain and skin, which are both ectodermal in origin, may be similar to each other but different from blood which is not ectodermal in origin. Thus, the ratio of full mutation to premutation allele in skin fibroblasts might be a better indicator of psychological impairment than the ratio in blood cells. © 1996 Wiley-Liss, Inc.

KEY WORDS: fragile X, mosaicism, adaptive behavior, tissue variation

INTRODUCTION

The fragile X syndrome is usually attributable to the expansion of a CGG triplet repeat in the 5′ end of the FMR1 gene and the associated methylation of a CpG island in this region [see Warren and Nelson, 1994 for review]. The syndrome results directly from the lack of FMR1 gene expression which is presumably due to the methylation of the CpG island [Pieretti et al., 1991; Sutcliffe et al., 1992]. FMR1 alleles are defined [Rousseau et al., 1991a] by the size of the triplet repeat region and the methylation of a cluster of CpG island restriction sites (e.g. Eag I) located approximately 220 base pairs (bp) 5′ to the CGG repeat [Fu et al., 1991]. Normal alleles with fewer than approximately 60 repeats and "premutation" alleles with 60 to 200 repeats are not methylated on an active X chromosome and express the FMR1 gene product at the same level [Feng et al., 1995]. "Full mutation" alleles with more than 200 repeats, are consistently methylated and have no FMR1 gene expression [Pierretti et al., 1991; Oberlé et al., 1991; Sutcliffe et al., 1992].

Mosaic fragile X individuals are defined by the presence of both premutation and full mutation alleles in leukocyte DNA [Rousseau et al., 1991a]. A premutation allele is detected in the blood DNA of 12 [Rousseau et al., 1994] to 41% [Nolin et al., 1994] of affected males and may actually be even more common [de Graaff et al. 1995a]. Mosaicism in females is detected at a lower level [Rousseau et al., 1994] presumably because methylation due to X inactivation reduces the apparent level of the premutation allele by approximately 50%.

Analysis of different fetal [Wöhrle et al., 1992] and adult [Yu et al., 1992; Taylor et al., 1994; de Graaff et al., 1995a] tissues indicates that different assortments of alleles may be present in different tissues of a full mutation individual. Since the fragile X CGG expansion probably occurs somatically during early embryogenesis [Devys et al., 1992; Wöhrle et al., 1993; Reyniers et al., 1993], the assortment of alleles in a tissue may depend on the stochastic path taken by the mutation in the embryonic progenitors of that tissue. Premutation size alleles in a tissue may represent an early stage of the expansion or may be deletion products of larger alleles. Premutation alleles are functional but some deletion alleles may not be [Hirst et al., 1995; de Graaff et al., 1995b].

One possible explanation for the wide range of psychological impairment in fragile X males is that the

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functional premutation allele present in many mosaics affects psychological function in some cases. Although two surveys of affected males [Rousseau et al., 1991a, 1994] did not find a correlation between IQ and mosaic status, recent studies [Cohen et al. and Wright-Talamante et al., these Proceedings] in which adaptive skills development and age were correlated with mosaicism, indicated that the behavior of mosaics, as a group, was less impaired than that of non-mosaic full mutation males.

To see if tissue variation could be a significant factor in mosaicism we analyzed the FMR1 gene in blood and skin samples in four fragile X mosaic males. We also determined their adaptive skills development to see if there was any indication that analysis of a skin sample might predict behavior. We were particularly interested in the skin samples because skin has a different embryonic origin from blood cells and is probably more closely related developmentally to the brain as both skin and nervous system tissue develop from the ectoderm. Buccal cell samples (from the lining of the cheek) which have an embryonic origin similar to skin were also analyzed in two of the cases.

METHODS AND SUBJECTS

Methods

Genomic Southern blot analysis was performed with EcoR I and Eag I and the probe StB12.3 [Rousseau et al., 1991a; Nolin et al., 1994]. Southern blots for subjects 3 and 4 were also hybridized with a probe from the 3' end of the FMR1 cDNA, BC22 [Verkerk et al., 1991] which identifies a 5 kb non-polymorphic EcoR I fragment, to control for variations in DNA migration. Primary fibroblast cell cultures were derived from skin biopsy samples from the lower forearm by standard techniques [Ausubel et al., 1993]. Fibroblasts were grown in culture until approximately 2×10^7 cells could be harvested for DNA analysis (3–4 passages). The buccal cell samples were collected with a toothbrush and DNA was isolated following standard techniques for cultured mammalian cells [Ausubel et al., 1993]. The relative amounts of pre- and full-mutation alleles in each sample was determined by densitometric analysis of autoradiograms. The density measurements were converted to percentages of pre- and full-mutation alleles by comparison to densities of a known mixture of pre- and full-mutation male DNA standardized by examination of a binary RFLP at another locus (DXS369).

Psychological evaluation was done independently and without knowledge of the DNA results. Adaptive skills data were obtained on all individuals by interviews with caregivers (usually the mother) using the Vineland Adaptive Behavior Scales [Sparrow et al., 1984]. The Vineland provides information about an individual's functioning in four behavioral domains: Communication; Daily Living Skills; Socialization; and Motor Skills. Standard scores and age equivalent scores can be determined for each domain. Additionally, overall adaptive behavior composite score can be determined to reflect each individual's general level of function.

Subjects

All four subjects were chosen by 1) classification as fragile X mosaics based on Southern analysis of genomic DNA from blood and 2) on the availability of skin fibroblast cultures. Subject 1 was evaluated at North Shore University Hospital. Subjects 2–4 were evaluated at NYS Institute for Basic Research in Developmental Disabilities. This study was approved by the Institutional Review Board of the NYS Institute for Basic Research in Developmental Disabilities and informed consent was obtained from all of the subjects or their guardians.

Subject 1 was 3 years 2 months at time of testing. He was an only child and the only member of his family with developmental delays. He was the product of a normal pregnancy and delivery. He was hypotonic in infancy. He sat alone between 5 and 7 months, crawled at 8 months, and walked at about 16 months of age. At 2½ years he had a mental age of 1 year 3 months on the Bayley Scale of Infant Development, Second Edition. He had a history of chronic ear infections and of seizures for which he was taking Tegratol. He was friendly with good eye contact. He had problems sustaining attention, was frequently aggressive (e.g., head butting and kicking), flapped his hands and spun his body and objects, flipped light switches on and off, needed routines, and was hypersensitive to loud noises and the sounds of running water and flushing toilets.

Subject 2 [the younger of the brothers studied in Brown et al., 1984] was 10 years 4 months at the initial time of testing. He was the product of a fraternal twin pregnancy. He had an older brother who was severely retarded and a twin sister with learning problems. This child was described as hypotonic and also experienced recurrent diarrhea. He sat at 6 months, walked at 15 months, and said his first word at 9 months. Mild symmetric hydrocephalus involving the lateral ventricles was noted on computerized axial tomography. He displayed gaze avoidance, perseverative language, echolalia, and would hyperventilate and become aggressive when introduced to change. He was also pleasant and friendly at times. At age 10 he obtained an IQ of 33 on the Stanford-Binet and at age 12 years 7 months he obtained an IQ of 33 on the Leiter International Performance Scale.

Subject 3 was 1 year 11 months at time of testing. He was an adoptee and there was no information about his family history. He sat at 6 months, crawled at 11 months, walked at 15 months, and said his first words at 13 months. At 1 year 5 months he had a mental age of 1 year 2 months on the Bayley Scale of Infant Development, Second Edition. He was diagnosed with external hydrocephalus and macrocephaly. He did not have seizures. He had a pleasant disposition and made good eye contact. He feared loud noises and would shriek and grind his teeth when bored.

Subject 4 was 2 years 2 months at time of testing. He was the second child in a family without any history of developmental delay. The child was healthy at birth, but developed an ear infection at 5 months. He sat at 7 months, crawled at 11 months, and walked at 16

months. At 2 years 1 months he had a mental age of 1 year 4 months on the Bayley Scale of Infant Development. He said his first words at 14 months. He did not have a history of seizures. His eye contact was very good. He displayed repetitive body movements when excited, hummed, smelled non-edible items, and stared at moving cars and leaves. In addition he feared loud noises, slept poorly, and would bite his hand when excited or aroused.

RESULTS

DNA Analysis

We used genomic Southern blot analysis to determine the proportion of full mutation and premutation alleles in different tissues of four fragile X mosaic males. The analysis in Figure 1 compared blood sample DNA and skin fibroblast DNA from subject 1. Examination of blood and skin DNA from this mosaic male indicated that there was a surprising difference between tissues: Approximately 60% of the blood cells carried a presumably functional premutation allele while >90% of the skin fibroblasts carried the full mutation.

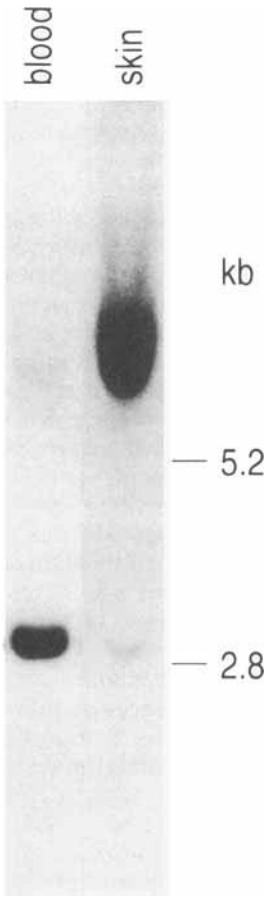

Fig. 1. Southern blot of subject 1 comparing genomic DNA from blood and skin fibroblasts. The premutation band which was pronounced in blood and barely detectable in skin was approximately 3.1 kb which represented an allele containing approximately 130 repeats. The full mutation alleles, which were >6 kb (>260 repeats, methylated), were barely detectable in blood but were pronounced in skin. The positions of the normal 2.8 kb band (30 repeats) and a 5.2 kb band representing the normal methylated allele in a female were determined in parallel lanes (not shown) and are indicated at the right.

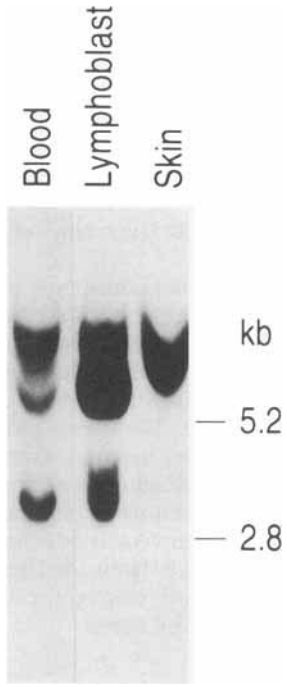

Fig. 2. Southern blot of subject 2 comparing genomic DNA from blood, cultured lymphoblastoid cells and skin fibroblasts. The premutation band in blood and lymphoblasts was approximately 3.2 kb (160 repeats). No premutation was visible in the skin sample even after extended autoradiography. The full mutation alleles were distributed between two major bands >6 kb (>260 repeats, methylated) in blood and lymphoblasts but only one compact array >7 kb (>560 repeats, methylated) was apparent in the skin sample. The markers are as in Figure 1.

The analysis in Figure 2 compared whole blood, lymphoblast, and skin fibroblast DNA from subject 2. Approximately 14% of blood (and lymphoblast) cells carried a premutation allele. In contrast no premutation allele was detected in the skin sample. This indicated that while a significant proportion of the blood cells in this mosaic male carried a premutation allele, most of the skin fibroblast cells carried a full mutation. As in subject 1, the proportion of premutation allele in skin was much different from blood.

The analysis in Figure 3 compared blood cell, skin fibroblast, and buccal cell DNA from subject 3. The blood DNA analysis showed that approximately 21% of the blood cells carried the premutation. Although the amount of DNA was reduced, the analysis of skin cells showed a similar distribution; approximately 20% of skin fibroblast cells carried a premutation allele also. The size of premutation and full mutation alleles in blood, however, appeared to be different from those found in skin. While both blood and skin had a 3 kb premutation band, blood had a second band at approximately 3.3 kb, while skin had a smaller band at ap-

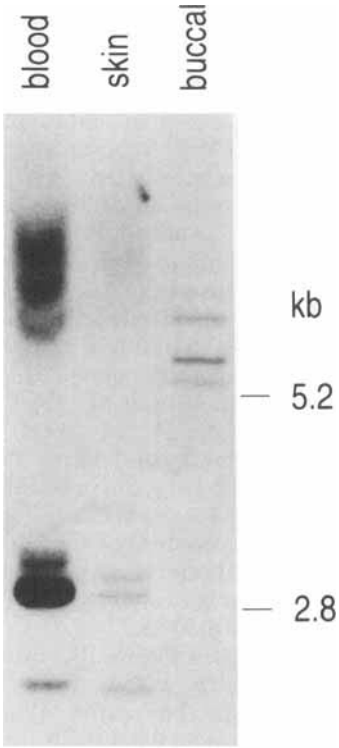

Fig. 3. Southern blot of subject 3 comparing genomic DNA from blood, skin fibroblasts, and buccal cells. In blood there was one major premutation band at approximately 3 kb (100 repeats) and minor bands at approximately 2.3 kb, which represented a deletion of 500 bp, and a second band at 3.3 kb (200 repeats) which represented another premutation allele. The full mutation alleles were a broad array of beginning at 6 kb (300 repeats, methylated). In skin (with less DNA in the sample) there were two premutation bands at approximately 3 and 3.2 kb (100 and 170 repeats, respectively) and a minor band, very similar in size to one seen in blood. In skin the full mutation array was larger than blood beginning at approximately 8 kb (1,000 repeats, methylated). In the buccal cell sample there was less DNA than in the skin sample. Three full mutation alleles were represented, a major band at approximately 6 kb (300 repeats) and minor bands at 5.7 and 7 kb (200 and 630 repeats, methylated). The markers are as in Figure 1.

proximately 3.2 kb. Blood and skin also showed a small band (<2.8 kb) which probably represented a deletion allele. Control hybridization of this blot (Methods) indicated that the observed differences in the band patterns were not due to differences in the quantity of DNA in each lane or other technical factors (data not shown). Although blood and skin patterns were different, the contrast between the relative amount of pre- and full-mutation alleles carried by blood and skin cells in this case was much less striking than the differences seen in Subjects 1 and 2.

Figure 3 also showed that buccal cells from subject 3 had a pattern of alleles that differed from both blood and skin. This pattern was dominated by a few distinct full mutation bands which had no strong counterparts in either skin or blood DNA patterns. The buccal cell DNA from subject 4 (not shown) also had a few distinct full mutation bands on a background pattern similar to that of skin and blood. The presence of a simplified pattern in the buccal samples suggested that these samples represented relatively few embryonic cells.

The analysis in Figure 4 compared blood cell, and skin fibroblast DNA from subject 4. Similar to subject 3, approximately 20% of the blood cells in subject 4 carried a premutation allele, while approximately 24% of skin cells carried a premutation. Subject 4 also had a different pattern in the skin fibroblast DNA; both the premutation and full mutation bands were different in size from those in blood. As in the analysis of subject 3, control hybridization indicated that the band pattern differences apparent in Figure 4, were not due to technical factors (data not shown).

Psychological Analysis

Each of the subjects was evaluated for his communication, daily living, socialization, and motor skills on the basis of caregiver interview. The composite Vineland Scale score from this information is listed with the rela-

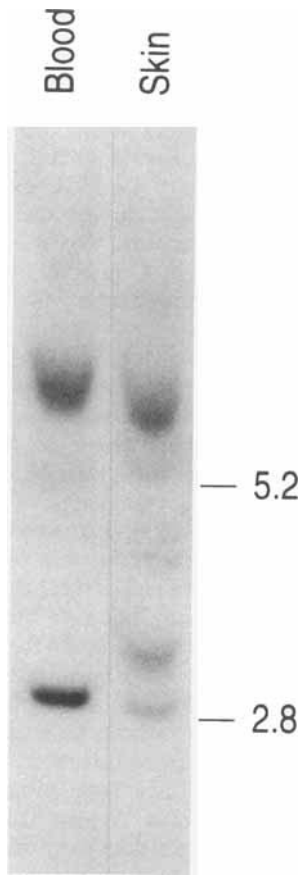

Fig. 4. Southern blot of subject 4 comparing genomic DNA from blood and skin fibroblasts. The premutation band in blood was approximately 3.2 kb (170 repeats). The full mutation alleles were distributed between a minor band at approximately 6 kb (300 repeats, methylated) and a major band >8 kb (1,000 repeats, methylated). In skin there were two premutation bands at approximately 3 and 3.3 kb (100 and 200 repeats, respectively). In skin the full mutation alleles were present primarily in two bands one at approximately 6.5 kb (460 repeats, methylated) and a band at approximately 7 kb (650 repeats, methylated). The markers are as in Figure 1.

TABLE I. Mosaicism in Blood and Skin Level of Adaptive Functioning

Subject	1	2	3	4
% Premutation:				
Blood	66	14	21	20
Skin	2	0	20	24
Vineland[a]	62	46	79	73

[a]Composite score with 100 = average.

tive representation of full and premutation alleles in skin in Table I. Subjects 1 and 2 in whom the full mutation dominates the mosaicism in skin fibroblasts scored lower on the Vineland Scale than did subjects 3 and 4, who have a readily detectable level of premutation in this tissue. As listed in Table I, the DNA results illustrate how much mosaicism can vary in different tissues *of a single* individual and how unpredictable this variation can be in different fragile X mosaics.

DISCUSSION

It has been inferred from the presence of different full mutation patterns in different tissues [Yu et al., 1992; Wöhrle et al., 1992: de Graaff et al., 1995a], from observations in twins [Devys et al., 1992], and from the presence of premutation sperm in full mutation males [Reyniers et al., 1993] that the CGG expansion probably takes place somatically during early embryogenesis. The results presented here, which show that different tissues in a fragile X mosaic may have very different proportions of the premutation allele, are consistent with this hypothesis.

Unlike the differences observed in full mutation patterns, the presence of a premutation-size allele in the array carried by a fragile X mosaic could have phenotypic consequences because these alleles are usually functional. The wide range in phenotype in fragile X is probably due, in part, to mosaicism of functional and silent alleles in many affected individuals. This is most obvious in full mutation females where X inactivation is responsible for mosaicism of functional and silent FMR1 alleles in each tissue. The random nature of X inactivation is presumably responsible for the phenotypic variation which even occurs in monozygotic female twins [Reiss et al., 1995]. The wide phenotypic range observed in males may similarly be due, in part, to the effect of a functional premutation in some mosaics. While fragile X mosaics are less impaired than nonmosaics [Cohen et al. and Wright-Talamante et al., these Proceedings], the studies here indicate that the relationship between mosaicism detected in blood cells and the phenotype may be indirect. Even though a functional, premutation, allele is present in many blood cells, it may not be present in many cells in the brain.

Differences between the mosaicism in the diagnostic tissue (blood) and the affected tissue (brain), however, may play a substantial role in masking the influence of mosaicism on impairment. The studies described here indicate that this difference between tissues can be extreme. Subjects 1 and 2 for example, show a very strong premutation band in blood but this functional-size allele is almost completely absent from the array in skin.

This tissue variation in fragile X mosaicism is presumably due to differences in embryonic origin. The brain originates from the embryonic ectoderm while blood cell precursors originate from a different embryonic source which may be determined very early—in the yolk sac or primitive streak [Bianchi et al., 1993]. Because of its embryonic origin, blood may be a poor representation of the assortment of FMR1 alleles in the brain of an affected individual. In addition the clonal expansion of leucocytes in immune response may select cells with a functional FMR1 gene, which apparently occurs in females [Rousseau et al., 1991b]. Thus, a diagnosis of mosaicism based on blood cell DNA may not be reliably predictive for an individual. The small number of subjects studied here did not allow us to infer how frequently there is a dramatic difference between blood and skin. It is possible that IQ scores and results with other behavioral and cognitive measures in a study of a larger group may show a clear relationship to the allele distribution in skin.

The buccal cell DNA in Figure 3 showed a simplified allele pattern in which a few intense bands were present. This suggests that a few alleles are represented in the sample taken from the lining of the cheek. Although it is not shown in Figure 4, a similar simplified full mutation pattern was also the case with Subject 4. This implies that the tissue sample represented a limited number of embryonic precursors. Thus, buccal cell samples may not reliably distinguish between premutation, mosaic, and full mutation individuals. A broader array of alleles was apparent in skin fibroblast samples. This suggests that fibroblasts may derive from a larger pool of embryonic cells. These results show that not only the size range but the number of different alleles may vary between tissues.

In conclusion the results presented here show that there can be a large difference in fragile X mosaicism in the blood and skin of a single individual mosaic. This variation is presumably generated by differences in FMR1 triplet repeat expansion in different cells in the early embryo. Since skin cells are developmentally more closely related to the brain than blood cells, mosaicism in skin may better represent the mosaicism in brain. Psychological evaluation of the four subjects in this study suggests that analysis of skin samples may predict the level of impairment of fragile X mosaic males. It will be necessary to study a larger group of fragile X mosaic males to see if there is a significant correlation between mosaicism in skin and the functional level of individual mosaics.

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